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Bacteria associated with compost used for cultivation of *Pleurotus tuber-regium* (FR.) Singer and *Lentinus squarrosulus* (Berk.), Nigerian edible mushrooms

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ABSTRACT: The bacteria involved in an outdoor single phase composting using saw dust and wheat bran as substrates for cultivation of *Pleurotus tuber-regium* (Fr.) singer and *Lentinus squarrosulus* (Berk.) Nigerian edible mushrooms were identified. Composting was carried out for 2 weeks. The highest core and peripheral temperatures were68 and 48° respectively while the lowest temperature was 32°C.

The highest number of bacteria in the core and peripheral compost were 1.46 x 10^6 and 6.90 x 10^5 cfu/ml respectively. Bacteria isolated and characterized from the fermenting agricultural substrates include *Bacillus polymyxa, Enterobacter aerogenes, Micrococcus roseus, Citrobacter freundii, Bacillus subtilois, Clostridium perfringens, Bacillus licheniformis, Pseudomonas aeruginosa, Bacillus cereus* and *Escherichia coli*. The implication of the presence of these bacteria is discussed.

Key words: Bacteria, Cultivation, Mushrooms, Compost, Fermentation.

Introduction

The first detailed record of mushroom cultivation occurred in A.D. 600 during the reign of Lious XIV when Tournefort described a successful method of growing mushroom *Agaricus bisporus* on stable manure. By the end of 18th century, composting using agricultural wastes as substrates for mushroom growing was recognized as essential tool for mushroom growers (Bahl 1988; Quimio *et al*., 1990).

Compost is a fertilizing mixture of partially decomposed organic matter from plant and animal origin (Piet *et al*., 1990). Composting is a solid-state fermentation process, which exploits the phenomenon of microbial degradation and mineralization (Mckinley and Vestal, 1984). The main purpose of composting to a mushroom grower is to prepare a substrate in which the growth of mushroom is promoted to the practical exclusion of other microorganisms. Fermor *et al*., (1985), reported that a composted substrate improved mushroom fruit body yield but, reduced infestation by insects, fungi and bacteria pathogens.

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Microorganisms colonizing mushroom compost during composting process are regarded as active agents which determine the chemical composition and mineralization thereby making it possible for mushroom growth (Fermor *et al*., 1985). Up till now, the identities of bacteria species involved in composts used for cultivation of *P. tuber-regium* and *L. squarrosulus* from Nigeria have not been well documented. Therefore, the objective of this present study is to improve information regarding those bacteria and proffer suggestions on how they could be used in a control fermentation process, which could further improve mushroom cultivation in Nigeria.

Materials and Methods

Sample Collection

The compost used in this study was prepared by using sawdust of *Terminalia superba* (locally known as 'afara' among the Yoruba people of South Western Nigeria). This was collected from Bodija Saw Mill, Ibadan, Nigeria and wheat bran (used as nutrient supplement) was obtained from the International Institute of Tropical Agriculture (IITA), Ibadan, Nigeria.

Composting

The compost was prepared by outdoor single-phase solid-state fermentation (Nair and Price, 1991). About 60.0kg of fresh sawdust were mixed thoroughly with 6.0kg (10% w/w) of wheat bran. Water was added until moisture content was between 40-60%. This is usually being determined by the 'rule of thumb' method (Buswell, 1984). The substrates were then stacked into heap of about 1.5m wide, 1.5m high and 1.5m long. This was covered with black polyethylene bags and left for 2 weeks with turning and re-stacking every 3-4 days to produce homogenous compost. Temperatures of the core and peripheral compost were measured twice daily (12.00 noon and 6.00 p.m.) during the period of the experiment. Likewise, fermenting compost samples were taken daily for bacteriological analysis.

Bacteriological Analysis

The procedure used involved placing 10.0kg of the compost sample with 90.0ml of sterile deionised water inside 250ml conical flask. This was shaken vigorously to form uniform solution of 10^{-1} concentration The stock was subjected to decimal dilution using sterile pipettes to form 10^{-2}, 10^{-3}. 10^{-4}, 10^{-5} and 10^{-7} concentrations using the method of Ejifor and Okafor (1985). These homogenate were used to determine different types of bacteria that were present in the compost and their colony-forming unit per milliliters (cfu/ml).

A Pasteur pipette was used to transfer 0.25ml of the last three dilutions on to a sterile plate of nutrient agar (NA) (Oxoid) and Eosine methylene blue agar (EMB) (Difco). The plates were agitated for even spread of the inoculum and incubated at 37°C for 24 hours. Colonies that appeared at the end of incubation were counted and unit expressed in term of colony forming unit per milliliter (cfu/ml). The distinct viable colonies were Gram stained and examined under oil immersion objective (x100). The colonies were then streaked on to appropriate agar to obtain pure cultures. Preliminary colonial morphology and microscopic examination were carried out on bacterial isolates. They were further subjected to standard biochemical tests shown on Table 2. Bacteria identification were carried out on the isolates by comparing the results obtained with the standard characterization definitions of Skermann (1967) and that of Bergey's manual of determinative systematic bacteriology (1986).

Results and Discussion

Ten bacteria species were isolated from saw dust/wheat bran based compost used in this study. They were coded isolates A, B, C, D, E, F, G, H, I and J (Tables 1 and 2). These microorganisms were characterized as *Enterobacter aerogenes, bacillus polymyxa, Micrococcus roseus, Citrobacter freundi, bacillus subtilis, Clostridium perfringes, Pseudomonas aeruginosa, bacillus cereus, bacillus licheniformis* and *Escherichia coli* (Table 2). These identifications were based on series of morphological and biochemical tests as well as physiological characteristics using the standard characterization definitions of Skermann (1967) and that of Bergey's manual of determinative bacteriology (1986).

The endospore forming rods, Gram positive, catalase positive that readily form chains ferment most sugars (Isolates B, E, H, and I) were identified as *Bacillus* species (Prescott *et al*., 1999). A simplified classification based on morphology, endospore position, starch and gelatin hydrolysis, motility, nitrate reduction and oxidative fermentation were used to classify Isolates B, E, H and I as *B. polymyxa, B. subtilis, B. cereus* and *B. licheniformis* respectively (Bergey's manua, 1986). The isolation of these bacteria from composting agricultural substrates suggests that a form of fermentation had taken place during the composting process. This is because Kolawole and Okonkwo (1985), Popoola and Ekueshi (1985), Okafor (1977) and Akinrele (1970) linked these organisms with fermentation of various agricultural substrates.

The bacterial cells that formed endospores and were oxidase negative and have anaerobic fermentative ability were identified as *C. perfringens* (Table 2). These bacteria had circular raised colony with 0.7cm diameter. They were non-motile, Gram-negative short rods. The isolation of *C. perfringens* agrees with reports of McKinley and vestal (1984) that composting is not a complete aerobic process. Therefore, anaerobic micro environment cannot be completely eliminated and the presence of non-oxidative bacteria cannot be avoided. The isolation of *bacillus* and *Clostridium* species from fermenting compost is not a surprise; this is because similar organisms were also isolated from fermenting cocoa beans (Rambouts, 1952; Ojey, 1981). Precott *et al*., (1999) also suggested that some *Bacillus* and *Clostridium* species inhabit high temperature habitats. Jones (1993), reported that these bacteria produce spores which are heat resistant thus making them to survive in an extremely high temperature of the compost. Similar reasons could be adduced for the presence of *Citrobacter freundi* and *Micrococcus roseus* since they are facultative anaerobes (Table 2).

Isolate J was indole positive, Gram-negative short rods that fermented lactose, maltose and fructose. Its growth on Eosine methylene blue agar to form Green metallic sheen was used to classify it as *E. coli* (Brock *et al*., 1986). *Enterobacter aerogenes* (Isolate A) was Gram-negative short rod. It has circular, convex and glistening colonies (Table 1). This organism which ferments lactose and fructose (table 2) was catalase positive and oxidase negative. The isolation of coliform group (*E. aerogenes* and *E. coli*) may be due to water source used in the mixing of the compost. The water might have been contaminated with these enterobacteriaceae. The bacteria were predominantly present at the early stage of composting process and died off when the temperature increased. The presence of *P. aeruginosa* (Isolate G) in the fermenting compost may be related to its ability to survive in vast number of habitats (Brook *et al*., 1986).

Table 3 shows that daily temperature of the core compost increased steadily from the 1st day (32°C) and attained it highest value (68°C) on the 5th day of composting process. A gradual temperature decrease was observedfrom the 6th day until the end of composting process (14th day). Ivors *et al*., (2000), observed a similar rise in temperature, while working on fermented agricultural substrates used for the cultivation of *Agaricus bisporus.*Carlile and Watkinson (1996), suggested that temperature had significant effect on the succession of microorganisms involved in fermentation process. Generally, the temperature of the corecompost was higher than the peripheral (Table 3). This is because of the core area of the compost is in a close state thereby restricting heat exchange and aeration (Jones, 1993).

The bacteria count of the core and peripheral compost is shown on Table 4. For the peripheral, there was an increase in bacteria count for the first 2 days. On the 3rd day, the value dropped significantly. This may be due to the confluent growth of *B. licheniformis* and *B. polymyxa*, which produce bacitracin and polymycin antibiotics, which could have inhibited the growth of other bacteria. The core area of the compost generally had higher bacteria count than peripheral because only few bacteria could survive high temperature observed in the core area. The available nutrients will be used for the growth of these few bacteria maximally with less competition.

Table 1: Colonial morphology of the bacteria isolated from fermenting compost.

	A	B	C	D	E	F	G	H	I	J
Shape	Circular	Irregular	Circular	Circular	Irregular	Circular	Irregular	Irregular	Rhizoid	Circular
Elevation	Convex	Flat	Raised	Raised	Raised	Raised	Raised	Raised	Raised	Convex
Size	0.4cm	1.5cm	0.2cm	0.7cm	1.0cm	0.7cm	0.4cm	0.4cm	0.5cm	0.1cm
Edge	Entire	Lobate	Entire	Erose	Erose	Entire	Serratia	Indulate	Lobate	Entire
Texture	Smooth/Glistening	Dry/Smooth	Smooth/Dry	Smooth/Glistening	Rough/Dry	Smooth/Glistening	Smooth/Glistening	Rough/Dry	Rough/Dry	Smooth/Dry
Consistency	Butryous	Rubbery	Butryous	Butryous	Butryous	Mucoid	Butryous	Rubbery	Rubbery	Butryous
Emulsification	Easy	Easy	Easy	Easy	Easy	Easy	Easy	Difficult	Difficult	Easy
Opacity	Opaque	Transparent	Opaque	Opaque	Opaque	Translucent	Translucent	Opaque	Opaque	Opaque
Chromogenicity	Yellow	Cream	White	Cream	White	Cream	Green	White	Yellow	Yellow

TABLE 2: BIOCHEMICAL PROPERTIES OF THE ISOLATES FROM THE MUSHROOM COMPOST

ISOLATE CODE	GRAM REACTION	CATALASE TEST	OXIDISE TEST	INDOLE TEST	METHYLRED TEST	VOGES – PROSKAUER TEST	MOTILITY	STARCH HYDROLYSIS	GELATIN HYDROLYSIS	CITRATE UTILIZATION	NITRATE REDUCTION	UREA HYDROLYSIS	HYDROGEN SULPHIDE PRODUCTION	SPORE STAINING	GLUCOSE	GALACTOSE	SUCROSE	LACTOSE	MANNITOL	REFFINOSE	MALTOSE	ARABINOSE	FRUCTOSE	CELLULAR MORPHOLOGY	PROPABLE ORGANISM
A	-	+	-	-	-	+	+	+	+	+	+	-	-	-	+	-	+	+	+	-	+	+	+	Rods	*Enterobacter aerogenes*
B	+	+	+	-	-	+	+	+	+	+	+			+	+	+	+	+	+	+	+	+	+	Rods	*Bacillus polymyxa*
C	+	+	+	-	-	-	+	-	-	-	+	+		-	+	-	-	-	+	+	-	+	-	Cocci	*Micrococcus roseus*
D	-	+	-	-	+	-	+	-	-	+	+	+		-	+	-	+	+	+	+	-	+	+	Short rods	*Citrobacter freundii*
E	+	+	+	-	-	+	+	+	+	+	+			-	+	-	+	+	+	+	-			Rods	*Bacillus substitis*
F	+	-	-	-	+	-	+	-	-	+	-			+	+	-	+	+	-	-	-	+	+	Rods	*Clostridium perfringens*
G	-	+	+	-	-	-	+	+	+	-	+	+	+		+	-	+	-	+	+	+	-	+	Rods	*Pseudomonas aeruginosa*
H	+	+	+	-	-	+	+	+	+	+	+			+	+	-	+	+	+	+	-	-	+	Rods	*Bacillus cereus*
I	+	+	+	-	-	+	+	+	+	+	+			+	+	+	+	+	+	-	-	-	-	Rods	*Bacillus licheniformis*
J	-	+	-	+	+	-	+	-	-	-	+	-	-	-	+	-	+	+	+	-	-	+	+	Short rods	*Escherichia coli*

Key

\- Represents Negative

\+ Represents Positive

Blank Represents Indeterminate Result

Table 3: Compost temperature during the period of fermentation

Day	12 Noon (°C)			
	Core	Peripheral	Core	Peripheral
1.	32	32	32	32
2.	46	44	47	43
3.	54	45	55	44
4.	65	46	65	46
5.	67	47	68	48
6.	64	45	63	44
7.	57	43	57	41
8.	53	38	55	38
9.	49	36	48	37
10.	45	36	46	35
11..	43	35	44	35
12..	42	34	43	33
13.	39	33	38	32
14.	34	32	33	31

Table 4: Viable bacteria count of peripheral and core area of the compost for 2 weeks.

Day	Colony forming unit per milliliter (cfu/ml)	
	Peripheral	Core
1.	6.0×10^6	6.0×10^4
2.	6.20×10^5	1.21×10^6
3.	6.90×10^5	1.46×10^6
4.	6.0×10^5	1.37×10^6
5.	5.8×10^5	9.3×10^5
6.	5.3×10^5	3.6×10^5
7.	4.9×10^5	3.2×10^5
8.	4.3×10^5	6.4×10^5
9.	1.68×10^5	1.6×10^5
10.	1.52×10^5	1.2×10^5
11.	1.60×10^5	1.04×10^5
12.	2.0×10^5	1.00×10^5
13.	1.85×10^4	0.96×10^4
14.	1.21×10^4	0.85×10^4

From the results obtained, it can be concluded that various bacteria genera are involved in the decomposition of compost making it suitable for the growth of *P. tuber-regium* and *L. squaurrosulus*. The pure cultures of these bacteria could be incorporated into agricultural wastes in a controlled fermentation unit. The next report will compare fruit body yield of *P. tuber-regium* and *L. squarrosulus* using composted and non-composted agricultural substrates.

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